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In Situ Biodegradation of Nitroaromatic Compounds in Soil

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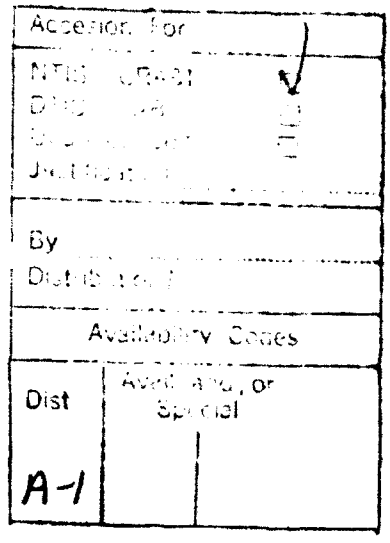

Anaerobic Degradation of 2,4,6-Trinitrotoluene (TNT) by a Defined Microbial Culture Isolated From an Anaerobic Bioreactor

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Introduction

In our ongoing research on the biodegradation of TNT by anaerobic bacteria, we have isolated a number of pure bacterial cultures from an anaerobic, methanogenic bench-top bioreactor that is fed a munitions extract containing TNT as the sole carbon and nitrogen source for growth of the anaerobic bacteria in the reactor. The defined microbial culture we have isolated from this bioreactor totally degrades TNT. Here, we summarize the results of this research.

Establishment of the Bench Top Bioreactor

The bioreactor was initially established with sewage sludge in 1989 and was supplemented with TNT and other munitions compounds as a sole source of carbon. These compounds were supplied in a defined medium containing essential nutrients. The culture present in the reactor adapted over time to utilize TNT, going through a number of sequential stages of adaptation characterized by the accumulation of specific TNT metabolites, which were identified by HPLC, GC/MS, and ^{14}C-radiolabeled tracer studies. The first metabolites to accumulate in culture supernatants were the reduced intermediates, 4-amino-2,6-nitrotoluene (4A26DNT) and 2,4-diamino-6-nitrotoluene (24DA6NT). A second stage of adaptation led to the disappearance of these intermediates and the accumulation of methylphloroglucinol (trihydroxytoluene). In a third stage of adaptation, trihydroxytoluene gave way to *p*-cresol (4-hydroxytoluene). After 3 years of selective pressure, the bioreactor accumulated acetate as the first identifiable non-aromatic TNT degradation product. When munitions wastes containing TNT, RDX, and HMX are fed to this adapted culture under optimized conditions for growth of the TNT-degrading consortium (final TNT concentration 125 mg/kg) the bioreactor accumulates detectable amounts of *p*-cresol in 14 days. This work will be presented in May 1993 at the annual meeting of the American Society for Microbiology (1) and in a paper to be published in Applied and Environmental Microbiology (2).

Isolation and Characterization of TNT-Degrading Microbial Culture (LJP 1) from the Bioreactor

Isolation of the Culture. The culture was isolated from the TNT degrading consortium present in the bench-top bioreactor. Its isolation was accomplished by serially diluting a sample from the bioreactor and plating the dilutions onto plates of Trypticase Soy Agar (TSA) supplemented with 5% (w/v) of Yeast Extract. This medium also contained 20 mg/L of TNT. After incubation isolated colonies were picked and restreaked on the same medium. Strictly anaerobic conditions were maintained by use of an anaerobic glove box. Using this procedure, a total of 12 isolates were obtained.

Initial Growth Experiments. Each of the 12 isolates was grown anaerobically in a minimal mineral salts medium containing 20 mM phosphate, carbonate buffer at pH 7-8, with TNT (30 mg/L) as the sole source of carbon and nitrogen for growth. The cultures were observed for growth (viable counts), and monitored for production of TNT metabolites by HPLC, using the method described by Funk *et al.* (2). Of the 12 isolates, only one, strain LJP 1, showed growth, elimination of TNT from the medium, and production of TNT degradation intermediates. Further work was performed with this strain only.

Strain LJP 1 was examined for its ability to grow anaerobically in a TNT minimal medium (mineral salts containing 30 mg/L TNT). Also included in the experiment was an inoculated control culture where LJP 1 was inoculated into the mineral salts broth not containing TNT or any other carbon source. LJP 1 inoculum was prepared by washing 24 hour old cells off of TSA+YE plates with mineral salts medium not containing TNT. Thirty ml of this cell suspension was used to inoculate 30 ml of the minimal medium to provide a final TNT concentration of 30 mg/L. The cultures were incubated standing at 30 C, and samples were removed at time 0, 12 and 48 hours. Viable counts were then determined for each sample on TSA/YE agar.

The time course viable plate counts showed a 3 log increase in cell numbers per ml after 48 hours growth in the minimal medium supplemented with TNT, whereas cell number declined from time 0 when TNT was omitted from the medium such that it contained no carbon source for growth. The results of this experiment are shown below in Table 1.

Table 1. Viable plate count results for growth of stain LJP 1 in the minimal medium with and without TNT (30 mg/L) as a sole source of carbon.

	Viable Count Colony Forming Units (CFU)/ml	
Incubation Time (Hr)	Medium -TNT X 10^6	Medium +TNT X 10^6
0	1.0	1.0
12	0.42	2.8
24	0.008	900
48	nd	1500

nd = not done

Identification of the Strain LJP 1. A Gram stain was run on a young, anaerobically grown culture of LJP 1, as was a spore stain. The stained cells were examined microscopically. In addition, cells were examined under a light microscope using wet mounts. This examination of the culture revealed the presence of large numbers of Gram positive, endospore-forming rods. LJP 1 was tentatively identified as a strain of *Clostridium*, although recent data shows that LJP 1 contains 5 bacterial members.

^{14}C-TNT Degradation and Mineralization Experiment. The growth experiments above had shown that during anaerobic growth LJP 1 removed TNT from the minimal growth medium, and produced TNT metabolites that were then also removed from the medium. There was also some indication of volatile organic acid production from the TNT. In light of these observations a TNT mineralization experiment was run using [U]-^{14}C-ring labeled TNT as a carbon source in the minimal medium. Serum bottles containing 100 ml of minimal medium (50 mg/L TNT) were inoculated with a 10% inoculum of cells, from TSA/YE plates, suspended in minimal medium. The medium also contained 208,000 DPM of ^{14}C-TNT (≥98% purity). The sealed cultures were then incubated anaerobically, without shaking, at 30 C. Cultures inoculated into the minimal medium containing TNT were run in duplicate (cultures A1 and A2). An uninoculated control was also run, as a single flask. Samples from each were removed after 24 hours, 4 days, and 7 days and analyzed by HPLC for presence of TNT, TNT degradation intermediates, and volatile organic acids (VOAs). ^{14}C present in each of the peaks eluted from the HPLC were quantified by liquid scintillation counting. Each culture was also flushed with nitrogen to recover CO_2 and volatile organic compounds which were trapped in the exiting gas using a trapping train consisting of 3 vials, first a scintillation cocktail (10 ml Biosafe scintillation cocktail)

to trap volatile organics, then two CO_2 traps in series (each containing 10 ml Biosafe scintillation cocktail with 1.0 ml of Carbosorb. ^{14}C present in cellular biomass was determined by collecting the cells from the medium by filtration and washing with first with water, then 10% trichloroacetic acid (TCA), then 5% TCA, and finally with methanol. The counts remaining on the filter were considered to be a component of the cellular biomass. The counts present in the water and TCA phases were also quantified

The results of this experiment are reported below for TNT mineralization, volatile organic compound formation, and accumulation of ^{14}C-labeled TNT degradation intermediates. Specific identities of the volatile organic acids produced from the ^{14}C-TNT are still being determined. These results are for cultures incubated for 24 hours, since results at days 4 and 7 were similar to those for 24 hours. As shown in Table 2, approximately 2% and 6% of the ^{14}C-TNT ring carbons were recovered as $^{14}CO_2$ and 4% and 5% as ^{14}C-volatile compounds in cultures A1 and A2 respectively. In addition, a minimum of 2-3% and possibly more of the label had been incorporated into cellular biomass. When combined with the viable plate count data, these total recoveries of 9%-13% of the ^{14}C as CO_2, volatile compounds, and/or biomass carbon show that the TNT is degraded and ultimately mineralized by LJP 1. The control showed no $^{14}CO_2$, ^{14}C-volatiles, or ^{14}C-biomass. Essentially all of the counts were recovered from as soluble counts in the culture medium.

In the control and inoculated cultures, after 24 hours a majority of the counts were recovered in the spent culture medium as soluble radioactivity (Table 2). Thus, it is important that their distribution in TNT and/or intermediates of TNT degradation be determined. Results from HPLC analyses of the control showed that after 24 hours, as the percentage of soluble radioactivity present, most counts (79%) were localized in 4-amino-2,6-dinitrotoluene (4A26DNT), an indication that essentially total abiotic chemical reduction of the 4-nitro group of TNT had occurred, probably due to the highly reducing conditions present in this medium since no TNT was detected by HPLC. Some counts (9% of the soluble counts) were also present in the peak corresponding to 2,4-diamino-6-nitrotoluene (24DA6NT), and in a peak corresponding to fraction 4, a 3.04 minute retention time peak representing 12% of the counts in an as yet unidentified intermediate. In inoculated culture A1, no radioactivity corresponding to the TNT peak and no TNT were detected in the culture supernatant. The peak representing 4A26DNT was reduced by 75% as compared to the control, and it contained 22% of the soluble radioactivity present in the supernatant. No 24DA6NT was present, although a small amount of radioactivity (3% of the soluble radioactivity) was present in fractions coming off at that retention time . The largest percentage of radioactivity (75% of the soluble counts) was found in the peak eluting with the void volume. Supernatants from inoculated culture A2 contained no TNT, either radioactive or not, while the 4A26DNT peak had been reduced by 81% as compared to the control. Approximately 24% of the soluble radioactivity was present in this peak. No 24DA6NT was detected, either radioactive or not. While no peaks were detected by HPLC at retention times between 7-8 minutes, some radioactivity (6% of the soluble counts) was present in fractions corresponding to this retention time. As with culture

A1, most of the soluble radioactivity (70%) eluted with the void volume of the column. In supernatants from the control and from both cultures A1 and A2, there was a large peak that eluted here. The identities of compounds eluting here have not been determined. They are, however, aliphatic degradation products. Overall, these results show that substantial TNT degradation, followed by removal of the TNT degradation intermediates was occurring in the inoculated cultures. Additional work will have to be completed to better quantify what is happening.

Table 2. Degradation of ^{14}C-ring labeled TNT by anaerobic bacterial culture LJP 1 after 24 hours of growth in the TNT-supplemented minimal medium as compared to an uninoculated control.

Fraction	% of ^{14}C Recovered (DPM)[a]		
	Uninoculated Control	Culture A1	Culture A2
CO_2	0	2.0	6.0
Volatile Organics	0	4.0	5.0
Biomass	0	3.0	2.0
Water Wash[b]	0	54.0	50.0
10% TCA Wash[b]	0	18.0	24.0
5% TCA Wash[b]	0	9.0	9.0
Methanol Wash[b]	0	0.1	16.0
Culture Medium[c]	100	2.0	2.0
Total Recoveries	100	101.1	104.0

[a] The total DPM per culture were 208,000

[b] These represent counts recovered upon washing the cells present on the filter. Since the extraction procedure was harsh, some of the counts (TCA and methanol washes) were likely biomass components (amino acids, etc.) removed from lysed cells.

Characterization of Microbial Culture (LJP 1)

Our most recent work shows that the TNT-degrading culture contains 5 different bacterial members. We have now isolated each of the strains. For these isolations, a loop full of cells from the culture (LJP 1) from a stock culture slant were inoculated into anaerobic Peptone-Pepticase-Yeast Extract (PPY) broth in a 50 ml flask and incubated anaerobically, with no shaking at room temperature until turbid (24 hr). Cells from this culture were then streaked onto solid medium (PPY agar) to isolate individual colonies. In this manner we isolated five pure bacterial cultures from LJP 1.

One pure culture isolated anaerobically from a single colony, is a rod-shaped bacterium (≈6.0μm X 1.1μm) producing ellipsoidal endospores. The sporangium is not swollen. The vegetative cells typically grow as single cells or in small chains. The culture is nonmotile. We have classified this culture as a strain of *Bacillus* or *Clostridium* and designated it as strain IMAGE 1. A second pure culture isolated anaerobically from a different colony is a rod-shaped bacterium (≈5 μm X 0.6 μm) which does not produce spores. The vegetative cell length varies whereas its width does not. This bacterium is Gram positive and appears to be nonmotile. We have tentatively identified this bacterium as a *Bacillus* species and designated it as strain IMAGE 2. A third pure culture was isolated anaerobically from the original culture slant. This was accomplished by heat shocking the slant at 65°C for 20 minutes, and then streaking the cells onto solid PPYA medium. From a single colony a pure bacterial culture was isolated. It is a rod-shaped bacterium (≈2.7 μm X 1.1 μm) growing in chains of up to 4 cells. it produces ellipsoidal spores that do not swell the sporangium. We have classified this bacterium as a *Clostridium* species and have given it the strain designation IMAGE 3. A fourth pure culture was isolated aerobically by streaking from the above broth culture onto PPYA medium. The bacterium is a catalase negative coccus (≈0.7 μm X 1.0 μm) which grows as single cells or in short chains. We have tentatively classified it as a species of *Streptococcus* and given it the strain designation IMAGE 4. A fifth pure culture isolated aerobically from the above broth culture as a single colony on PPYA medium is a Gram positive, catalase positive coccus (0.8 μm X 1.3 μm), and cells grow as small clusters. The bacterium is β-hemolytic. We have classified it a species of *Staphylococcus* and have designated it as strain IMAGE 5.

The five strains described above are summarized below in Table 1.

Table 1. Identities of the five bacterial strains isolated from TNT-degrading microbial culture LJP 1.

Strain Designation	Properties	Classification
1) IMAGE 1	Size: 6 μm X 1.1μm Endospores: Ellipsoidal Sporangium: Not Swollen Shape: rod Motility: Nonmotile Anaerobic Growth: + Aerobic Growth: ?	*Bacillus* or *Clostridium*
2) IMAGE 2	Size: 5μm X 0.6μm Endospores: + (aerobically) ?? Endospores: - (anaerobically) ?? Shape: rod Growth: Single cells Cell length varies, width does not Motility: Nonmotile Anaerobic Growth: + Aerobic Growth: ?	*Bacillus*
3) IMAGE 3	Size: 2.7 μm X 1.1 μm Endospores: Ellipsoidal (0.7 μm X 1.5 μm) Sporangium: Not Swollen Shape: rod Growth: Single cells/Short chains Motility: Nonmotile Anaerobic Growth: + Aerobic Growth: -	*Clostridium*
4) IMAGE 4	Size: 0.7 μm X 1.0 μm Catalase: - Endospores: None Shape: coccus Growth: Single cells/Short Chains Motility: Nonmotile Anaerobic Growth: + Aerobic Growth: +	*Streptococcus*
5) IMAGE 5	Size: 0.8 μm X 1.3 μm Catalase: + Endospores: None Shape: coccus Growth: Small Clusters Motility: Nonmotile Anaerobic Growth: + Aerobic Growth: + β-hemolytic	*Staphylococcus*

Degradation of TNT by the Pure Culture Isolates

It is important that we determine the relative role of each member of culture LJP 1 in the degradation of TNT. Now that the cultures are available, we have begun experiments to determine how each organism metabolizes TNT.

Conclusions

1) The Anaerobic bench-top bioreactor contains an anaerobic bacterial consortium that is capable of utilizing TNT as a sole source of carbon and nitrogen.

2) A defined culture, LJP 1, was isolated from the bioreactor and shown capable of degrading an mineralizing TNT in a minimal medium containing TNT as the sole carbon source for growth.

3) Culture LJP 1 consists of 5 members, including one strain of *Bacillus* or *Clostridium* (IMAGE 1) , one *Bacillus* strain (IMAGE 2), a *Clostridium* (IMAGE 3), a *Streptococcus* (IMAGE 4), and a *Staphylococcus* (IMAGE 5).

4) The roles of each bacterial member of LJP 1 in TNT degradation remains to be elucidated.

References

1) Funk, S.B., D.J. Roberts, D.L. Crawford, and R.L. Crawford. 1993. Degradation of Trinitrotoluene (TNT) and Sequential Accumulation of Metabolic Intermediates by an Anaerobic Bioreactor During its Adaptation to a TNT Feed. To be Presented, Annual Meeting American Society for Microbiology, Atlanta, GA. May 16-21.

2) Funk, S.B., D.J. Roberts, D.L. Crawford, and R.L. Crawford. 1993. Initial Phase Optimization for the Bioremediation of Munitions-Contaminated Soils. In Press, Appl. Environ. Microbiol.

University of Idaho
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ANAEROBIC BIOREMEDIATION OF SOILS CONTAMINATED BY MUNITIONS RESIDUES

Technology Developed by:	University of Idaho, Moscow, ID
Patent Pending:	Licensed to the J. R. Simplot Company, Pocatello, ID
Implementation Partners:	Envirogen, Inc., Lawrenceville, NJ, and Bioremediation Services, Lake Oswego, OR
Contact :	Dr. Ronald L. Crawford, University of Idaho, Center for Hazardous Waste Remediation Research , FRC 202, Moscow, ID 83844-1052, tel., 208-885-6580, FAX, 208-885-5741.

PROCESS DESIGN

Soil contaminated by munitions residues, particularly 2,4,6-trinitrotoluene (TNT) and RDX, is excavated, crushed if needed, screened (1/4 inch), supplemented with a starchy carbon source (1-2% by weight of soil), and mixed with phosphate buffer (50 mM) at pH 7-8. Soil/water ratios are 70/30 to 50/50 (weight/volume) depending on the soil. Also, depending on the nutritional status of a soil, small amounts of other nutrients (most often ammonium chloride as an initial nitrogen source) may be added. Metabolism of the added starch by indigenous microorganisms results in rapid consumption of oxygen, driving the redox potential to -200 mV or lower. Fermentation processes then lead to the destruction of munitions residues in about 20-30 days at 25-30°C. Initial transformations include reduction of nitro groups of TNT to amino groups, replacement of the amino groups with hydroxyl groups followed by replacement of the hydroxyl groups by hydrogen atoms. Key intermediates appear to be 2,4-diamino-6-nitrotoluene (2,4-DAT), phloroglucinol (2,4,6-trihydroxytoluene) and *p*-cresol (4-methyl phenol). The cresol ultimately is fermented to volatile organic acids, particularly acetate. All toxic aromatic structures are removed, with complete cleavage of benzene rings and no accumulation of the polymeric materials seen in most aerobic or microaerophilic treatments of TNT. RDX , HMX, and other munitions contaminants also are removed.

PROCESS ADVANTAGES

- Complete destruction of TNT, RDX, HMX and other contaminants without accumulation of toxic products or polymeric materials
- Simple engineering; no requirements for vigorous stirring or oxygenation; use of simple reactors for small sites, or pit configurations for large sites
- No increase in bulk as with composting
- Readily available, inexpensive supplements (starchy agricultural wastes, ammonium chloride)
- Relatively short incubation times (20-30 days); treatments accomplished in one season
- Wet conditions decrease explosion hazards
- Microbiology and catabolic pathways understood and predictable
- Toxicity of TNT to microbes limited by its moderately low solubility (TNT dissolution occurs during biodegradation)
- Significantly less expensive than available alternatives
- Available for immediate full-scale implementation by licensee and contracting partners